Final Report

Original 2 of 2

Determination of Skin Irritation Potential of [redacted]
in the Human Skin Model Test
following EU-Method B.46

Study No. [redacted]

Sponsor:
[redacted]

Test Facility:
[redacted]

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1 GLP-Compliance Statement

It is hereby declared that all tests were made in accordance with the „Revised OECD Principles of Good Laboratory Practice" (Paris, 1997) as stated in the following guidelines:

- OECD Principles of Good Laboratory Practice, adopted by Council on 26th November 1997; Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (codified version)
- Chemikaliengesetz (Chemicals Act) of the Federal Republic of Germany (ChemG) §19a and §19b and annexes 1 and 2 in the version of 02 July 2008 published in Bundesgesetzblatt No. 28/2008, pp. 1146 - 1184

There were no circumstances that may have affected the quality or integrity of the study.

[redacted]

Study Director

[redacted]

Date

Information on Study Organisation:

Deputy Study Director

Study Plan dated

Experimental Starting Date

Experimental Completion Date

Draft Report dated

[redacted]

2 QUALITY ASSURANCE UNIT STATEMENT

This study has been inspected by the quality assurance unit according to the principles of Good Laboratory Practice. Study Plan and Final Report were checked at the dates given below, the Study Director and the management were informed with the corresponding report.

Also, the performance of the study was inspected, and findings were reported to Study Director and management. The inspection of short-term studies (duration less than four weeks) is carried out as audit of process concerning major technical phases of at least one similar test. Frequency is once or more a quarter.

The study was conducted and the reports were written in accordance with the Study Plan and the Standard Operating Procedures of the test facility.

Deviations from the Study Plan were acknowledged and assessed by the Study Director and included in the Final Report.

The reported results reflect the raw data of the study.

Verified Procedure	Inspected on	Findings reported on	Audit report no.
Study plan	[redacted]		
Performance of study			
Draft report			
Final report			

[redacted]

Table of Contents

3 SUMMARY

Title of Study: Determination of Skin Irritation Potential of [redacted] in the Human Skin Model Test following EU-Method B.46

Findings and Results:

One valid experiment was performed.
Three tissues of the human skin model EpiDerm™ were treated with [redacted] for 60 minutes.

In average, 26.5 mg of the solid test item (wetted with 25 µL DPBS-buffer) were applied to each tissue and spread to match the tissue size.

DPBS-buffer was used as negative control, 5% SDS-solution was used as positive control.

After treatment with the negative control, the absorbance values were within the required acceptability criterion of 1.0 < mean OD < 2.5. The positive control showed clear irritating effects. Variation within tissues was acceptable (< 18%).

After the treatment with the test item, the relative absorbance values were reduced to 94.4 %. This value is well above the threshold for irritation potential (50%). Therefore, [redacted] is considered as not irritant in the Human Skin Model Test.

4 PURPOSE OF THE STUDY

This in-vitro study was performed in order to evaluate the potential of [redacted] to evoke skin irritation in a human-skin-model.

The test consists of a topical exposure of the neat test item to a human reconstructed epidermis model followed by a cell viability test. Cell viability is measured by dehydrogenase conversion of MTT (3-[4,5-dimethyl thiazole 2-yl] 2,5-diphenyl-tetrazoliumbromide), present in cell mitochondria, into a blue formazan salt that is quantitatively measured after extraction from tissues. The percent reduction of cell viability in comparison of untreated negative controls is used to predict skin irritation potential.

Sponsor's intent: REACH.

5 LITERATURE

The study was conducted in accordance with the following guidelines:

- Commission Regulation (EC) No. 761/2009, Method B.46: "In Vitro Skin Irritation: Reconstructed human epidermis model test" adopted 23. July 2009
- OECD Guideline for the Testing of Chemicals, Draft Proposal for a New Guideline, *In Vitro* Skin Irritation: Reconstructed Human *Epidermis* (RhE) Test Method, Version 7.6, 9 September 2009
- ECVAM international validation study on *in vitro* tests for acute skin irritation: Report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test (Altern Lab Anim. 2007 Dec; 35 (6): 559-601).
- Protocol for IN VITRO EpiDerm™ SKIN IRRITATION TEST (EPI-200-SIT), Rev. 3/23/2009, MatTek Corporation, Ashland, MA 01721, USA

[redacted]

6 MATERIALS AND METHODS

6.1 Test Item

6.1.1 Specification

The following information concerning identity and composition of the test item is provided by the sponsor.

Name
Batch no.
Appearance
Composition
CAS No.
EINECS-No.
Molecular formula
Molecular weight
Purity
Homogeneity

[redacted]

Vapour pressure
Stability
Solubility
Production date
Expiry date
Storage
Hazard information
R-phrases
S-phrases

[redacted]

6.1.2 Storage
The test item was stored in a closed vessel at room temperature.

6.2 Negative Control

Dulbecco's Phosphate Buffered Saline (DPBS buffer without $CaCl_2$ and without $MgCl_2$). Composition see chapter 6.5.4

6.3 Positive Controls

Sodium dodecylsulphate, CAS No. 151-21-3, solution in deionised H_2O containing 5% SDS. Procured by Mattek Corporation, batch: 030310TVKD.

6.4 Test System

6.4.1 Specification
Commercially available Epi-200-SIT-Kit.

The EpiDerm™ tissue consists of normal, human-derived epidermal keratinocytes which have been cultured to form a multi-layered, highly differentiated model of the human epidermis. It consists of organized basal, spinous and granular layers, and a multi-layered stratum corneum containing intercellular lamellar lipid layers arranged in patterns analogous to those found in vivo. The EpiDermTM tissues are cultured on specially prepared cell cultures inserts.

6.4.2 Origin
Epi-200 tissues were procured from MatTek Corporation in Ashland, USA.

Day of delivery: 06. Oct. 2010, batch: 13830.

6.5 Chemicals and Media

6.5.1 MTT Medium
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (=MTT), which can be reduced to a blue formazan. Prepared by [redacted]

A MTT stock solution of 5 mg/mL was prepared and stored in aliquots of 2 mL at – 20 °C, batch no.: 20100914. 2 mL of the stock solution were thawed and diluted with 8 mL of the Assay medium (resulting in 1 mg/mL). This MTT-solution with the concentration of 1 mg/mL was used in the irritation test; batch no.: 20101009.

6.5.2 Assay Medium
Serum-free DMEM medium. Procured by MatTek Corporation, batch: 093010TTE

6.5.3 Isopropanol
CH_3-CH(OH)-CH_3, p.A., 99.9 %, batch: 1620853, used as extracting solvent for formazan

6.5.4 DPBS-Buffer

Solution for the rinsing of the tissues (also used as negative control). A subset was procured by MatTek Corporation; the other subset was prepared by [redacted]

Composition of the subset from MatTek Corporation, batch no.: 090710aca:

KCl	0.2 g
KH_2PO_4	0.2 g
NaCL	8.0 g
$Na_2HPO_4 * 7H_2O$	2.16 g
H_2O	ad 1 L

Composition of the subset from [redacted]

KCl	0.2 g
KH_2PO_4	0.2 g
NaCL	8.0 g
$Na_2HPO_4 * 2H_2O$	1.44 g
H_2O	ad 1 L

The buffer which was procured by MatTek Corporation was used as negative control and for rinsing the test item from the tissues. The buffer which was prepared by [redacted] was used for rinsing the outside of the inserts at the end of the incubation time with MTT only.

6.6 Test Vessels

All vessels used are made of glass or sterilizable plastic. They were sterilised before use by heating to 180 °C (two hours) or autoclaving.

The following vessels were used:

- Schott-bottles
- 500 mL plastic wash bottle
- beakers 200 mL and 400 mL
- 6-well-plates, 24-well plates, 96-well-plate
- Glass funnel
- Small glass weight boards

6.7 Instruments and Devices

The following instruments and devices were used in the performance of the study.

- SANYO Labo Autoclave MLS 3020
- Stop-watch
- 96-well-plate photometer MRX with a 570 nm filter
- Precision scales Mettler PB 5001-S02
- Analytical scales Mettler Toledo AB 184 SA
- Incubation chamber Binder No.9, adjustable to 37 °C, 5% CO_2
- Table water bath GFL
- Adjustable pipettes with sterile tips:
  [redacted] (200 - 2000 µL), [redacted] (20 - 200 µL)
- Laminar flow bank
- Forceps, blunt-edged
- Mortar & pestle
- Orbital shaker GFL 3005
- Bulb-headed pipettes, sterile
- Nylon mesh circles 8 mm diameter (200 µm pore (EPI-MESH))

Usage and, if applicable, calibration of all instruments following the corresponding SOP in the current edition.

7 PERFORMANCE OF THE STUDY

The solid test item was ground.

7.1 Pre-Tests

First, the test item was tested for forming a colour without MTT addition. 27.2 mg were given in a test tube with 0.3 mL H_2O demin. and incubated at 37 °C and 5% CO_2 for 60 minutes. The resulting solution was colourless.

Then, the test item [redacted] was tested for the ability of direct formazan reduction. To test for this ability, 24.6 mg were added to 1 mL of MTT reagent and the mixture was incubated in the dark at 37 °C for 60 minutes. Untreated MTT reagent was used as control. The MTT reagent didn't change its colour within one hour, therefore, direct MTT reduction had not taken place, and no data correction was necessary.

7.2 Pre-Incubation of Tissues

Eight 6-well-plates were prepared with 0.9 mL assay medium in three of the six wells (upper row). The tissues were inspected for viability. Viable tissues were transferred (three per plate) in the wells with the medium using sterile forceps under the laminar air flow bank and placed into the incubator at 37°C and 5% CO_2 for one hour.

After the pre-incubation (one hour), the other three wells of each plate (lower row) were filled with fresh assay medium (0.9 mL). Every tissue was transferred into a well of the lower row. All 6-well-plates were set into the incubator at 37°C and 5% CO_2 for 18 hours. The Assay medium was stored in the fridge.

7.3 Treatment

The pre-incubated tissues were placed into fresh 6-well-plates containing 0.9 mL assay medium per well, using the upper row only.
One plate (three tissues) was used as negative control; each tissue was treated with 30 µL DPBS buffer. One plate was used as positive control; each tissue was treated with 30 µL SDS-solution. One plate was used for treatment with the test item. The tissues were wetted with 25 µL DPBS buffer before applying the test item and spreading it to match the tissue size.

The following amounts were applied to the plate:

Table 7.3-a Amounts of Test Item

Tissue	Amount
1	27.7 mg
2	27.5 mg
3	24.3 mg

Tissues were dosed in one minute intervals. After dosing the last tissue, all plates are transferred into the incubator for 35 min.. 60 min. after the first application, the inserts were removed from the plates using sterile forceps and rinsed immediately in one-minute-intervals.

Dosing of first tissue:	11:00 h
Dosing of last tissue:	11:17 h
Start of incubation at 37 °C:	11:25 h
End of incubation at 37 °C:	12:00 h
Rinsing of first tissue:	12:01 h
Rinsing of last tissue:	12:20 h

After rinsing, each tissue was dried with a sterile cotton tip and then transferred into a new 6-well-plate with fresh assay medium (0.9 mL). The tissue surfaces were evaluated visually under the stereo microscope, excess test item was removed, where necessary.

Then the tissues were set in the incubator for 24 hours.

7.4 Medium Renewal

For three incubated tissues, a new 6-well-plate with 0.9 mL assay medium in the upper row was prepared. The tissues were removed from the incubator and shaken for ten min (500 rpm). Then the inserts were transferred into the new 6-well-plate and set into the incubator for 18 ± 2 hours for post-incubation.

The medium from the "old" 6-well-plates was collected in the labelled 24-well-plate. It can be stored for 12 months at – 20 °C for possible interleukin analysis.

7.5 MTT Assay

After a total incubation time of 42 hours, a 24-well-plate was prepared with 300 µL freshly prepared MTT-reagent in each well. The tissues were blotted on the bottom and then transferred into the 24-well-plate. Then the 24-well-plate was set into the incubator for 3 hours ± 5 min.

After this time, the MTT reagent was aspirated and replaced by PBS buffer. This was then aspirated, too, and replaced several times. At last, each insert was thoroughly dried and set into the empty, pre-warmed 24-well-plate. Into each well, 2 mL isopropanol were pipetted, taking care to reach the upper rim of the insert. The plate was then shaked for two hours at room temperature.

After two hours, the inserts in which formazan had been produced were pierced with an injection needle, taking care that all colour was extracted. The inserts were then discarded and the content of each well was thoroughly mixed in order to achieve homogenisation.

From each well, two replicates with 200 µL solution (each) were pipetted into a 96-well-plate which was read in a plate spectral photometer at 570 nm.

8 EVALUATION

The values of the 96-plate-reader were transferred into a spreadsheet (Microsoft Excel®).

The photometric absorption of the negative controls was considered as 100%. For the mean of the two replicates of test item and positive control, formazan production was calculated as % photometric absorption compared with the negative control.

8.1 Calculations

The photometric absorption of the negative controls is considered as 100%. For the mean of the three replicates of test item and positive control, formazan production is calculated as % photometric absorption compared with the negative control:

$$\%\ \text{Formazan production} = \left[\frac{\text{OD}_{\text{test item}}}{\text{OD}_{\text{negative control}}}\right] \cdot 100$$

8.2 Assessment

Skin irritation potential of the test item is assessed as given in the following table:

Table 8.2-a Assessment of Irritation Potential

% Formazan production	Assessment
< 50% of negative control	Irritant
> 50% of negative control	Non-irritant

9 FINDINGS AND RESULTS

9.1 Measured Values

As blank, the optical density of isopropanol was measured in eight wells of the 96-well-plate. The measured values and their mean are given in the following table:

Table 9.1-a Absorption values blank isopropanol (OD at 570 nm)

Replicate	1	2	3	4	5	6	Mean
Absorption	0.070	0.100	0.082	0.078	0.095	0.107	**0.089**

The absorption values of negative control, test item and positive control are given in the following table:

Table 9.1-b Absorption Values negative control, test item and positive control (OD at 570 nm)

Designation	Measurement	Negative Control	[redacted]	Positive Control
Tissue 1	1	2.411	2.094	0.240
	2	2.444	2.083	0.239
Tissue 2	1	2.023	2.077	0.244
	2	2.040	2.084	0.247
Tissue 3	1	2.289	2.183	0.255
	2	2.261	2.216	0.256

From the measured absorptions, the mean of each tissue was calculated, subtracting the mean absorption of isopropanol as given in table 9.1-a. Mean and relative standard deviation (comparison of the three tissues) were also calculated.

Table 9.1-c Mean Absorption Values

Designation	Negative Control	[redacted]	Positive Control
Mean – blank (Tissue 1)	2.339	2.000	0.151
Mean – blank (Tissue 2)	1.943	1.992	0.157
Mean – blank (Tissue 3)	2.186	2.111	0.167
Mean of the three Tissues	**2.156**	**2.034**	**0.158**
Relative Standard Deviation of the three tissues	9.3%	3.3%	5.1%

9.2 Comparison of Formazan Production

For the test item and the positive control, the following percentage values of formazan production were calculated in comparison to the negative control:

Table 9.2-a % Formazan Production

Designation	[redacted]	Positive Control
% Formazan production (Tissue 1)	92.8%	7.0%
% Formazan production (Tissue 2)	92.4%	7.3%
% Formazan production (Tissue 3)	97.9%	7.7%
% Formazan production Mean	**94.4%**	**7.3%**

9.3 Assessment and Validity

9.3.1 Irritation Potential of the Test Item

The relative absorbance values were reduced to 94.4% after the treatment. This value is above the threshold for irritation (50%). Therefore, the test item is considered as not irritant.

9.3.2 Validity and Acceptability

Validity criteria and results are stated in the following table:

Table 9.3-a Validity

Criterion	Demanded	Found
OD of negative control	between 1.0 and 2.5	2.156
% Formazan production of positive control	≤ 20% of negative control	7.3%
Variation within replicates (RSD)	< 18%	9.3 % (negative control) 5.1 % (positive control) 3.3 % (test item)

The value for the negative control was within the range of historical data of the test facility (see annex 2, page 19).

The value for the positive control was not within the range of historical data. This is considered as uncritical for the following reason: The deviation was only 6.4% below the respective range of the historical data (lowest value in history was 7.8 %, value found in this study: 7.3%) and within a range of mean ± 2 standard deviations. Variation of biological systems within this order of magnitude is not unusual; furthermore, for the preparation of different batches of the cell cultures, different cell donors may have been used.

Therefore, the experiment is considered valid.

10 Discussion

The test item is considered not irritant.

After the treatment, the relative absorbance values were decreased to 94.4%. This value is well above the threshold for irritation (50%).

The optical density of the negative control was well within the required acceptability criterion of 1.0 < mean OD < 2.5. The positive control induced a decrease in the relative absorbance as compared to the negative control to 7.3% (required: ≤ 20%) for thus ensuring the validity of the test system. Variation within replicates was within the accepted range for negative control, positive control and test item.

For these reasons, the result of the test is considered valid.

11 Deviations

11.1 Deviations from the Study Plan

The following deviations from the study plan were observed:

- The plate was not left to stand over night at room temperature. The plate was shaken for two hours and then the tissues were pierced with an injection needle. This deviation was signed as uncritical because the [redacted] describes this method, too.

The deviation was signed by the deputy study director on [redacted]

- Application: 25 mg were demanded in the study plan. For the test for colour formation without MTT addition (pre-test), 27.2 mg test item was used. In the experiment, 26.5 mg (average) were applied. This deviation was signed as uncritical since it is not always possible to weigh and apply a test item exactly to 1.0 mg.

The deviation was signed by the deputy study director on [redacted]

11.2 Deviations from the Guideline

None as known.

12 Recording

One original of study plan and final report, respectively, all raw data of the study and all documents mentioned or referred to in study plan or final report will be kept in the GLP Document Archive of the test facility for fifteen years. After that, the sponsor's instructions will be applied (shipment of documentation to sponsor). A retain sample of the test item will be kept in the GLP Substance Archive for fifteen years; then, the retain sample will be discarded.

Number of originals which will be sent to the sponsor: 1

13 ANNEX 1: COPY OF GLP CERTIFICATE

RheinlandPfalz

Gute Laborpraxis / Good Laboratory Practice

GLP-Bescheinigung / Statement of GLP Compliance

(gem. /according to § 19 Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 88/320/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 88/320/ EEC at:

Prüfeinrichtung / Test facility

[redacted]

Prüfung nach Kategorien / Areas of Expertise
(gem. / according ChemVwV-GLP Nr. 5.3/OECD guidance)
1, 3, 4, 5, 6, 8

Datum der Inspektion / Date of Inspection
(Tag.Monat Jahr / day.month year)
27. und 28. November 2006

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.
Eine erneute behördliche Überprüfung der Einhaltung der GLP-Grundsätze durch die Prüfeinrichtung ist so rechtzeitig zu beantragen, dass die Folgeinspektion spätestens vier Jahre nach dem Beginn der o.g. Inspektion stattfinden kann. Ohne diesen Antrag wird die Prüfeinrichtung nach Ablauf der Frist aus dem deutschen GLP-Überwachungsprogramm genommen und diese GLP-Bescheinigung verliert ihre Gültigkeit:

Based on the inspection report it can be con- firmed, that the test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Verification of the compliance of the test facility with the Priciples of the GLP has to be applied for in time to allow for a follow-up inspection to take place within four years after commencing the above mentioned inspection. Elapsing this term, the test facility will be taken out of the German GLP-Monitoring Programme and this GLP Certificate becomes invalid.

Unterschrift, Datum / Signature, Date

11.01.2007

Dr.-Ing. Karl-Heinz Rother - Präsident -
(Name und Funktion der verantwortlichen Person / name and function of responsible person)

Siegel

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht
Kaiser-Friedrich-Straße 7
55116 Mainz

(Name und Adresse der GLP-Überwachungsbehörde / Name and adress of the GLP Monitoring Authority)

Landesamt für Umwelt, Wasserwirtschaft und Gewerbeaufsicht

[redacted]

14 ANNEX 2: COMPARISON WITH HISTORICAL DATA

In the following table, the mean of the negative controls and positive controls of all performed experiments up to [redacted] is stated and compared with the values which were found in this study.

Table 14-a Historical Data

Parameter	Optical Density Negative Control	% Formazan production Positive Control
Mean	1.766	11.1%
Standard Deviation	0.338	3.5%
Range	1.347 – 2.254	7.8 – 17.1%
Study [redacted]	**2.156**	**7.3%**